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14. ABSTRACT

The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million affected U. S. citizens and active service members. Development of in vivo therapies that restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. Thus, characterizing ancestral regulatory circuitry controlling regeneration is a necessary and direct route to identifying the mechanistic causes of regenerative failure in mammals. This proposal offers unique promise in guiding the targeted development of in vivo therapies to restore/augment human limb regeneration. We will leverage our recent discovery that regenerative ability is widespread in basal vertebrates to conduct the first comparative analysis of appendage regeneration that incorporates model systems from all major groups of limbed vertebrates- cartilaginous fishes, ray-finned fishes, and tetrapods. Our unique approach will identify novel functional requirements for genes/gene networks in regulating appendage regeneration by marrying a comparative organismal approach with state-of-the-art systems-level analyses of gene expression using next generation RNA sequencing and functional analysis of candidate regulators in the genetically tractable zebrafish model system through in vivo disruption of gene function.

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THE CENTER FOR REGENERATIVE BIOLOGY AND MEDICINE AT MOUNT DESERT ISLAND BIOLOGICAL LABORATORY

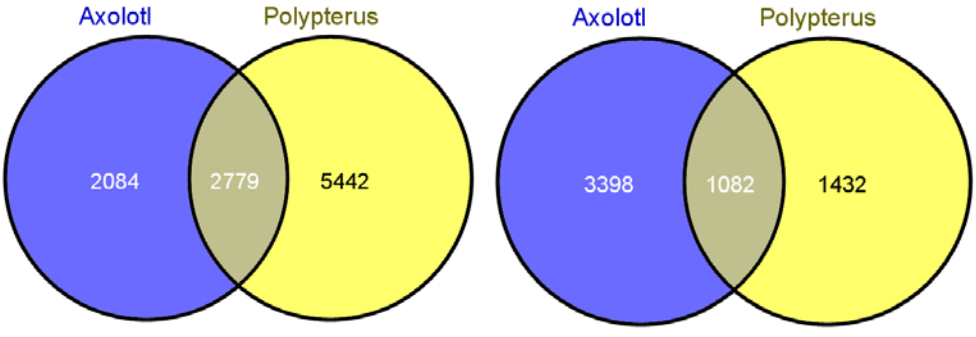
INTRODUCTION:

The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million affected U. S. citizens and active service members. Development of *in vivo* therapies that restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. Thus, characterizing ancestral regulatory circuitry controlling regeneration is a necessary and direct route to identifying the mechanistic causes of regenerative failure in mammals. This proposal offers unique promise in guiding the targeted development of *in vivo* therapies to restore/augment human limb regeneration. We will leverage our recent discovery that regenerative ability is widespread in basal vertebrates to conduct the first comparative analysis of appendage regeneration that incorporates model systems from all major groups of limbed vertebrates- cartilaginous fishes, ray-finned fishes, and tetrapods. Our unique approach will identify novel functional requirements for genes/gene networks in regulating appendage regeneration by marrying a comparative organismal approach with state-of-the-art systems-level analyses of gene expression using next generation RNA sequencing and functional analysis of candidate regulators in the genetically tractable zebrafish model system through *in vivo* disruption of gene function.

BODY:

Specific Aim 1: Characterize mRNA expression profiles in regenerating salamander (Ambystoma mexicanum) limbs and Polypterus senegalus fins. This work will be accomplished by a) collecting tissue and mRNA from regenerating salamander and *Polypterus* appendages, b) characterizing gene expression profiles by DNA sequencing using Solexa/Illumina technology, c) assembling and annotating transcriptome sequence, d) identifying conserved regulators of limb/fin regeneration by quantitative bioinformatics analysis, and e) assessing functional requirements of candidate regulators.

Research Accomplishments: The major focus for Aim 1 was to identify regulatory programs essential for injury induced regeneration of limbs/appendage tissues in *Ambystoma* (axolotl) and *Polypterus* animals. The defining feature of limb/appendage regeneration is the formation of a highly proliferative structure called a blastema. This structure is formed through dedifferentiation of tissues proximal to the amputation plane and serves as a source for all regenerating tissues. We reasoned that mRNAs that are important for blastema formation are likely to be differentially regulated in response to injury. Thus, for each species, we compared and contrasted the dataset between uninjured and 7 days post-amputation (dpa) samples, a stage during regeneration with a well-defined blastema. To further refine the genes important for regeneration, we applied another selection factor to identify those genes that were similarly controlled in both *Polypterus* and *Ambystoma* samples. These comparisons revealed a total of 2779 shared genes that were significantly upregulated during regeneration. Conversely, our analysis showed that 1082 genes were downregulated in



UPREGULATED **DOWNREGULATED**

Figure 1: Venn diagram of UniProt protein sequence IDs among Axolotl and Polypterus contigs that were up-regulated and down regulated greater than 2-fold between 0 and 7 dpa. (Left) The common set of 2,779 upregulated UniProt protein sequence IDs were mapped to 2,617 human Ensembl genes. (Right) The common set of 1,082 downregulated UniProt protein sequence IDs were mapped to 1,019 human Ensembl genes.

response to limb amputation (Figure 1).

From this dataset, we identified the 10 most highly upregulated genes during regeneration in both *Polypterus* and *Ambystoma* limb regeneration (Table 1). This collection of genes is involved in remodeling of the extracellular matrix, a process that is critical during cell movement and cellular dedifferentiation. In the absence of cellular remodeling of the epidermal tissue, mesenchymal cells proximal to the amputation plane do not undergo cellular dedifferentiation and therefore, heal injuries with defective blastema formation.

Table 1. Top ten most highly upregulated genes during axolotl and *Polypterus* limb regeneration.

Associated Gene	Description
KRT19	keratin 19 [Source:HGNC Symbol;Acc:6436]
MMP9	matrix metallopeptidase 9 [Source:HGNC Symbol;Acc:7176]
CTSK	cathepsin K [Source:HGNC Symbol;Acc:2536]
MMP13	matrix metallopeptidase 13 (collagenase 3) [Source:HGNC Symbol;Acc:7159]
PRDM1	PR domain containing 1, with ZNF domain [Source:HGNC Symbol;Acc:9346]
MMP10	matrix metallopeptidase 10 (stromelysin 2) [Source:HGNC Symbol;Acc:7156]
MMP1	matrix metallopeptidase 1 (interstitial collagenase) [Source:HGNC Symbol;Acc:7155]
NCF2	neutrophil cytosolic factor 2 [Source:HGNC Symbol;Acc:7661]
MMP3	matrix metallopeptidase 3 [Source:HGNC Symbol;Acc:7173]
KRT17	keratin 17 [Source:HGNC Symbol;Acc:6427]

Since genome databases for *Ambystoma* and *Polypterus* are not available, we also queried our transcriptome dataset for homologues of known genetic markers of blastema formation. To perform these studies we used the well-characterized zebrafish caudal fin appendage system as a reference for regeneration. These previous zebrafish studies identified the cell cycle regulator (*mps1)*, the Fibroblast growth factor (*fgf20a)*, transcription factors (*junb)* and (*smarca4)*, matrix metalloproteinase (*mmp9)* and chemokine (*cxcl12a)* as genetic determinants of blastema formation. We identified these regulators in both the *Ambystoma* and *Polypterus* genomes and validated their expression changes with real-time qPCR studies (Figure 2). These blastemal genetic markers in *Ambystoma* and *Polypterus* show similar expression changes to the zebrafish genes, implicating them as important modulators of blastema formation (Figure 2). In summation, work accomplished in Aim 1 revealed a subset of differentially regulated genes that are potentially vital for the formation of the regenerative blastema.

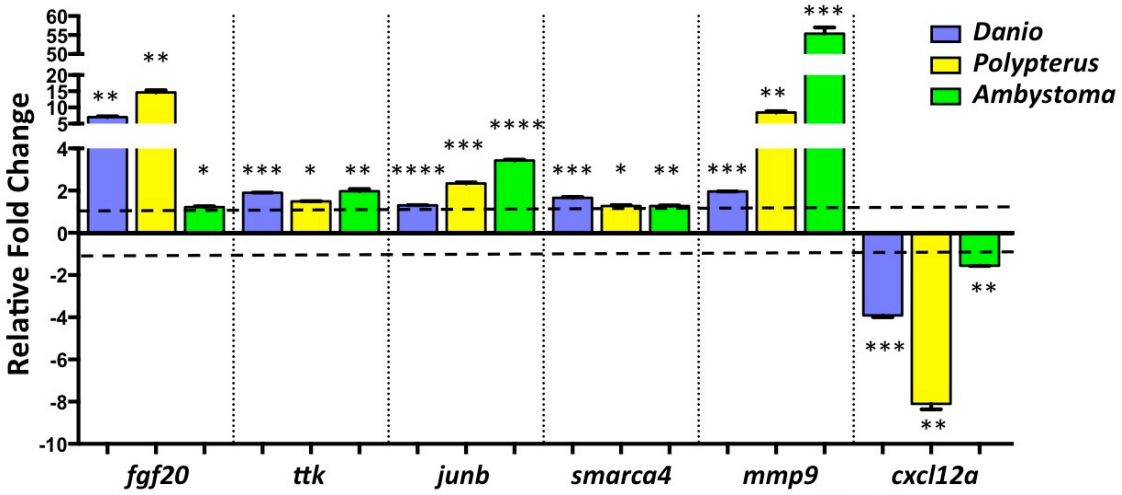


Figure 2: Conserved Patterns of miRNA Expression in Blastema Tissues. qRT-PCR validation of differential expression of commonly up-regulated (A) and down-regulated (B) miRNAs in regenerating zebrafish caudal fins (0dpa vs. 4dpa), Polypterus pectoral fins (0dpa vs. 7dpa) and axolotl forelimbs (0dpa vs. 7dpa).

Specific Aim 2: Characterize microRNA (miRNA) expression profiles in regenerating salamander (*Ambystoma mexicanum*) limbs and *Polypterus senegalus* fins. This work will be accomplished by **a**) collecting tissues and isolating small RNA from regenerating salamander and *Polypterus* appendages, **b**) characterizing miRNA expression profiles generated by DNA sequencing using Solexa/Illumina technology, **c**) annotating miRNAs and characterizing their expression profiles in limb/fin regeneration, **d**) predicting miRNA targets and correlating miRNA expression with predicted targets, and **e**) assessing functional roles of candidate miRNAs.

Research Accomplishments: Regeneration of complex appendage tissue requires differentiated, quiescent tissues being transformed into proliferative, progenitor cells that accumulate to form the blastema. This naturally reprogrammed, indispensable progenitor tissue is the source of all regenerating appendage tissue, including connective tissue, nerves, blood vessels, pigment cells and epidermis. Blastema formation requires the rapid modulation of genetic programs.

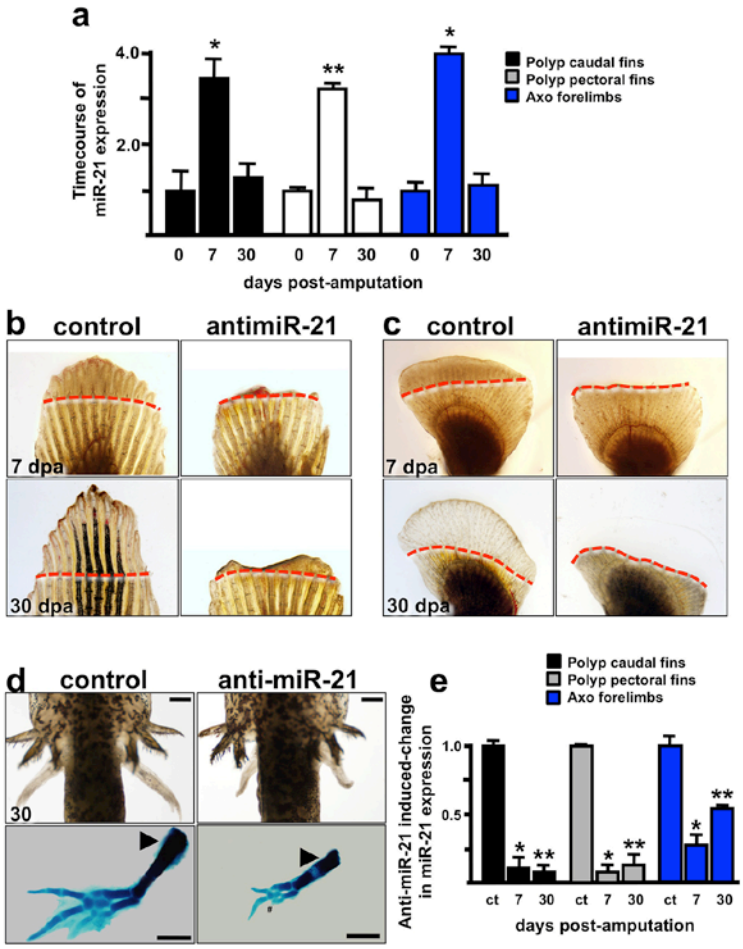


Figure 3. miR-21 is required for axolotl and Polypterus limb regeneration. A) Real-time qPCR studies reveal the temporal expression profile of miR-21 during appendage regeneration. **B-C)** *Polypterus* caudal fin (B) and pectoral fin (C) regeneration in control and antimiR-21 treated animals. (Red dashed line = original amputation plane). **D)** Limb regeneration in axolotl treated animals. (Arrowhead = original amputation plane). E) Real-time qPCR studies showing changes in miR-21 levels after LNA-antimiR-21 treatment.

Gene regulatory networks are controlled at multiple levels. miRNAs are key regulatory factors during gene expression with the unique ability to modulate hundreds of target genes, thus making them ideal candidates to control the cellular process of complex tissue regeneration. We isolated total RNA, enriched for small miRNAs (less than 200 nucleotides) and performed deep sequencing for small RNAs. Our analyses identified 8 different miRNAs, including miR-21, miR-181a, miR-7a, let-7j, miR-130c, miR-338, miR-204 and miR-2184 that are shared between regenerating *Ambystoma* forelimbs, zebrafish caudal fins and *Polypterus* pectoral fins. miR-21 was the most highly upregulated miRNA in all three model systems examined. In the absence of miR-21 activity, appendage regeneration was defective in both zebrafish and *Polypterus* fin appendages, and axolotl limbs (Figure 3). *Polypterus* caudal and pectoral fins exhibited no regeneration due to failure in blastema formation. In axolotls however, the primary role for miR-21 appears to be pattern formation. miR-21 depleted animals regenerate shorter and thicker limbs when compared to control animals (Figure 3). In short, our studies indicate that miR-21 is an essential, conserved component of the regenerative genetic circuit.

To define the potential mechanism of action for miR-21 function in response to appendage injury, we first used real-time qPCR studies to identify changes in genetic markers of regeneration in the zebrafish caudal fins. Consistent with a strong defect in regenerative outgrowth, the blastema marker *msxb*, the cell cycle regulator *mps1* and the ETS transcription factor *pea3* were all downregulated when miR-21 activity was depleted (Figure 4). Interestingly, key components of the Fibroblast growth factors (Fgf) were elevated in expression despite the strong block in regeneration. We were surprised to observe significantly elevated levels of *fgf20a* and *mkp3* given that increased expression is associated with normal regenerative outgrowth of appendage tissue (Figure 4A). How can this paradoxical result between gene expression of regeneration markers and the lack of regeneration be explained?

One possible explanation is that miR-21 fine-tunes Fgf expression levels, keeping its activity within a permissive window that promotes cellular dedifferentiation (Figure 4C). Appendage regeneration and limb development are particularly sensitive to Fgf expression levels. For instance, previous zebrafish appendage regeneration studies showed that mutations in *fgf20a* inhibit blastema formation and regenerative outgrowth. Likewise, inhibition of the entire Fgf signaling network with activation of the *Tg(hsp70:DN-Fgfr1)* transgene, culminated in regeneration inhibition. Conversely, elevated Fgf levels during development are inhibitory, functioning to terminate limb bud outgrowth. Since, *fgf20a* has a predicted binding site for miR-21 in the 3' UTR, we propose that miR-21 is a critical regulator of Fgf activity during tissue repair and regeneration. Upon appendage injury, the normal upregulation of miR-21 dampens the sudden increase in Fgf activity, thus positioning Fgf expression within a conducive window for tissue repair and regeneration (Figure 4C).

Growth factor sequester model: In this alternative sequester model, we hypothesize that growth factors like *fgf20a* are being activated normally in response to injury but are sequestered due to inhibition of extracellular matrix (ECM) remodeling by decreases in matrix metalloproteinase (MMPs) activity (Figure 4D). MMPs are normally held in an inactive state through their association with tissue inhibitors of MMPs (timps). Disrupting the MMP/timp balance is critical for remodeling of ECM space in order to promote cellular migration and proliferation and cell-cell communication during development.

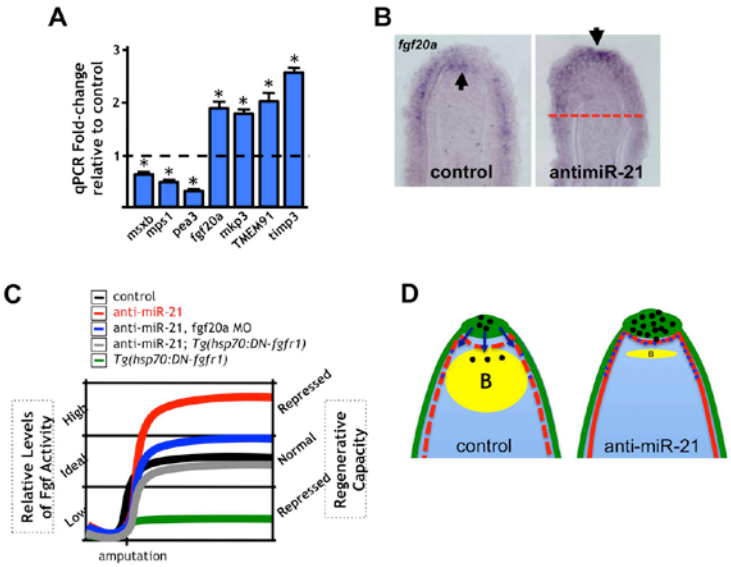


Figure 4. Two models of miR-21 mechanism of action. A) Real-time qPCR studies show decreases in *msxb*, *mps1* and *pea3* and elevation of *fgf20a*, *mkp3*, *TMEM91* and *timp3* expression levels. **B)** *In situ* hybridizations of control and antimiR-21 fin sections to detect *fgf20a* expression. Arrowhead represent *fgf20a* expression and red dashed line = original amputation plane. **C)** A depiction of the Growth factor threshold model. Fgf activity must be tightly controlled and maintained in an "ideal" range for regeneration to proceed normally. Low or high levels of Fgf represses regeneration. **D)** An illustration of the Growth factor sequester model. Fgf20a (black circles) migration is essential to activate blastema (yellow circle with B) formation. Fgf20a ligand passage to the mesenchyme requires changes to cellular adhesion and ECM remodeling of the basal-epidermis tissue (red line). These remodeling events of the basal-epidermis are inhibited under conditions of miR-21 depletion. (* = Student's ttest p-value <0.05).

We predict that under normal regeneration conditions, *fgf20a* activation is localized to the wound epidermis and then disperses into neighboring proximal cells whereby it induces cellular dedifferentiation and subsequent blastema formation. However, migration of *fgf20a* requires remodeling of the basal epidermal tissue, which lies between the wound epidermis and the mesenchyme. As cellular dedifferentiation progresses, levels of *fgf20a* are diminished through interactions with miR-21. In *Tg(hsp70:miR-21^{sp})* or antimiR-21 treated animal, *in situ* hybridizations studies demonstrate that indeed *fgf20a* expression is confined to the thickened epidermis (Figure 4B). Furthermore, two validated miR-21 target genes, tissue inhibitor of metalloproteinase 3 (*timp3*) and ECM transmembrane factor TMEM91, are highly enriched in appendage tissues devoid of miR-21 activity (Fig. 3A). In addition, preliminary studies with a pan-cadherin antibody to detect intercellular adhesion suggest that animals lacking miR-21 activity have tighter cellular association and are devoid of changes in cellular morphology within the basal-epidermal tissue layer. Collectively, we believe these results suggest that miR-21 may promote dispersal of growth factor ligands via remodeling of the ECM of the basal-epidermis tissue. We are working to test these two models of miR-21 mechanism of action with various cellular and molecular approaches.

Specific Aim 3: Determine a genetic positional memory code for appendage regeneration. This work will be accomplished by **a**) isolating tissue and RNA from uninjured and regenerating zebrafish caudal fins, **b**) profiling miRNAs with microarray hybridization technology and **c**) filter and categorize the dataset into increasing and decreasing miRNA expression.

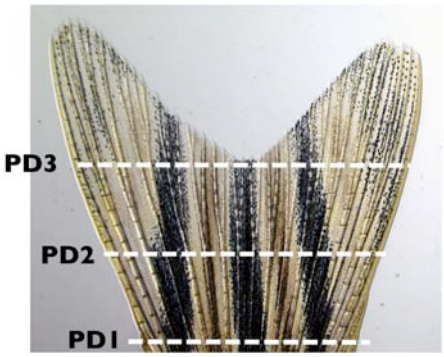


Figure 5. Schematic of positional memory amputation planes. Adult zebrafish caudal fins were independently amputated at proximodistal 1-3 (PD1-3). A second amputation plane, located at ~ 2 bony segments proximal to the original cut sites, were harvested for total RNA isolation.

Research Accomplishments: To initiate our studies on positional memory, we isolated uninjured tissue from specified regions of the zebrafish caudal fin. The amputation planes were designated proximodistal (PD) 1-3 (Figure 5). We collected tissue that was ~2 bony segments directly proximal to the PD amputation plane and extracted total RNA. To achieve our research goal of identifying candidate miRNAs as regulators of positional memory, we also collected 4 dpa regenerating tissues in a similar manner. All collections were performed in triplicate. Toward our research goal of identifying candidate miRNAs as regulators of positional memory, we performed RNA sequencing experiments to identify changes in small RNA expression at the different amputation planes. Our initial analysis indicated that a subset of miRNAs is differentially expressed at specific planes when comparing the uninjured samples to the regenerating groups (Table 2). miR-21 was the most highly upregulated miRNA in response to amputation at all injury planes; and therefore, was chosen for functional studies.

Table 2. miRNA changes at different amputation planes.

UPD1 vs RPD1 Upregulated	UPD1 vs RPD1 Downregulated	UPD2 vs RPD2 Upregulated	UPD2 vs RPD2 Downregulated	UPD3 vs RPD3 Upregulated	UPD3 vs RPD3 Downregulated
dre-miR-21	dre-miR-738	dre-miR-21	dre-miR-101a	dre-miR-21	dre-miR-139
dre-miR-181a*	dre-miR-2190	dre-miR-181a*	dre-miR-2184	dre-miR-181a*	dre-miR-2190
dre-miR-451	dre-miR-204	dre-let-7i	dre-miR-204	dre-miR-15c	dre-miR-738
dre-miR-2188	dre-miR-101a	dre-miR-181b	dre-miR-338	dre-miR-15b	dre-miR-101a
dre-miR-31	dre-miR-2184	dre-miR-15c	dre-miR-29b	dre-miR-140*	dre-miR-338

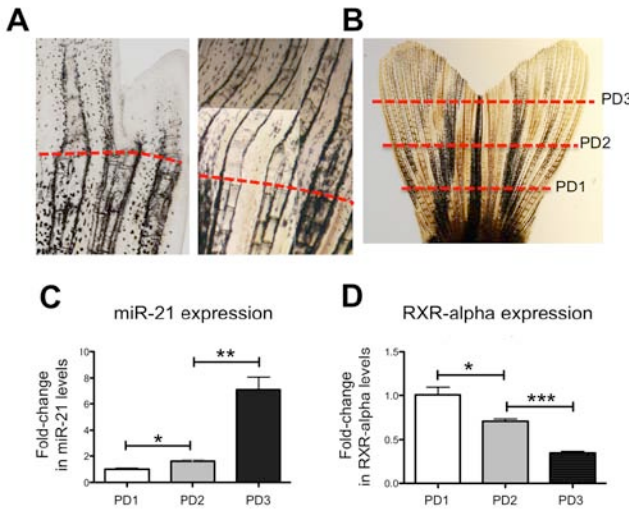


Figure 6. Loss of miR-21 phenocopy retinoic acid activity overexpression. A) Regenerating caudal fins treated with antimiR-21 depletion (Left) or retinoic acid treatment[1] (Right). **B)** Proximal-distal amputation planes in the caudal fin. **C-D)** Real-time qPCR studies show the regionalized expression of miR-21 or *RXR-alpha* during caudal fin regeneration. (*,**, *** = Student's ttest p-value <0.01, 0.005, 0.001).

Specific Aim 4: Define requirements for region-specific regulatory factors in maintaining positional memory. This work will be accomplished by **a**) validating miRNA expression using Northern blot hybridization and/or real-time quantitative PCR, **b**) determining spatial resolution of miRNAs during regenerative states and **c**) performing functional studies on miRNAs using antisense oligonucleotides to determine the effects on regeneration.

Research Accomplishments: In our functional studies we noted that animals with mild miR-21 inhibition regenerated appendage tissues with altered patterning. This defect in fin bone bifurcation patterning was similar to results observed in appendages treated with exogenous retinoic acid (RA) (Figure 6A). Given these similarities, we hypothesized that miR-21 may control RA activity during regenerative tissue patterning. First, we asked if miR-21 and RA expression was regionalized during appendage regeneration. We divided the caudal fin into three zones along the proximal-distal axis, termed PD1-3, to represent defined areas of the caudal fin. We used a razor blade to remove 75%, 50% or 25% of the caudal fin (Figure 6B). We believe incorporating three different amputation planes enhances the probability of identifying genetic factors that are expressed in a gradient than previous work that relied solely on two planes of injury. For intact appendage samples, we collected tissues 2-bony segments proximal to this initial amputation plane. In studies of regeneration, we collected the regenerated tissues at 4 dpa because it

represents a stage in appendage regeneration when positional cues have been determined. RNA was isolated from uninjured and regenerating tissues using Tri-reagent in accordance to the manufacturer's protocol and used for real-time qPCR studies (Sigma). Under conditions of no injury and during tissue regeneration, our studies showed that miR-21 expression is inversely expressed with components of the RA pathway, including *raldh2*, *RXR-α* and *RXR-Δ*. Whereas miR-21 expression is low at PD1, components of the RA pathway show elevated expression levels (Figure 6C, D). Most importantly, the dataset suggested that positional identity is maintained under conditions of homeostasis and re-established in freshly injured appendage tissue and miR-21 may be a critical component of this regulatory network.

Specific Aim 5: Grow Mount Desert Island Biological Laboratory as a collaborative center for the Regenerative Biology and Medicine research community. This work will be accomplished by establishing a MDIBL Visiting Scholars Program in Regenerative Biology.

Research Accomplishments: During this grant period, we have recruited and awarded visiting scientist fellowships to leaders in the field of regenerative biology and to scientists who have helped to grow the Mount Desert Island Biological Institute. All awards were made to applications focused on deciphering cellular processes during development and regulation of stress biology. Recent studies have shown that these cellular processes are key to understanding how the regenerative genetic circuit is activated. Below are the scientists that were awarded fellowships during the course of this grant. These investigators all delivered a scientific seminar during their time at MDIBL.

1. Ken Poss, Ph.D., Duke University Medical Center: Heart regeneration in the zebrafish.
2. Larissa Williams, Ph.D., Bates College: The importance of transcription factors in the Nfe2 family in the embryonic response to environmental toxicant exposure.
3. Zoya Ignatova, Ph.D., University of Potsdam, Germany: The effect of intrinsic (repetitive mutations and aging) and extrinsic (environmental stress) stress on translation dynamics.
4. Jorge Contreras, Ph.D., New Jersey Medical School: The role of Connexin channels in development and normal organ function.
5. Malcolm Maden, Ph.D., University of Florida: Appendage regeneration and reactive oxygen species.

Additionally, we also established a one-of-a kind 2-week comparative regeneration biology short course that provides laboratory intensive training for undergraduates, Ph.D. candidates and assistant professors. This included students from Australia, New Zealand, Sweden, South America and across the USA. This new course brought together leading scientists and students to study and discuss fundamental questions in regeneration biology and its practical application. Extensive hands-on laboratory and bioinformatics exercises form the core of the course. Within this dynamic environment each student: 1) examined, characterized and compared regenerative potential across a wide array of species; 2) gained practical guidance regarding animal care, handling and husbandry; 3) combined microsurgical methods with state of the art molecular analysis; and 4) joined a growing network of colleagues studying regeneration. A unique feature to the program was the incorporation of comparative bioinformatics approaches throughout the course to identify key common regenerative signatures between species. A key aspect of this course was the successful recruitment of leaders in the regenerative field such as Drs. Alejandro Sanchez Alvarado, Brigitte Galliot, Ken Poss, David Stocum and Karen Crawford to be the core course faculty. In total, our efforts to establish the MDI Biological Laboratory as a collaborative center for regenerative biology reached across the globe during the duration of this grant-funding period.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified miR-21 as the most highly upregulated miRNA in Axolotl, *Polypterus* and zebrafish limb injuries.
- Demonstrated that miR-21 is required for blastema formation and regenerative tissue patterning in all three limb/appendage regeneration systems.
- Identified a miR-21-retinoic acid genetic axis for positional memory and tissue patterning during zebrafish appendage regeneration.

- Successfully recruited and awarded regeneration fellowships to prominent regeneration scientists.
- Established a unique laboratory intensive comparative regeneration biology short course that was taught by the leaders of the regenerative biology field.

REPORTABLE OUTCOMES:

Poster presentation and abstract at the 3rd North Atlantic Zebrafish Research Symposium:

miR-21 is an evolutionarily conserved regeneration miRNA

Heather Carlisle, Ashley Smith, Benjamin King and Viravuth P. Yin
Davis Center for Regenerative Biology and Medicine, Mount Desert Island Biological Labs, Salisbury Cove, ME 04672, USA

Appendage regeneration is defined by the transformation of quiescent, differentiated tissues into highly proliferative and regenerative blastemal cells. These dramatic cellular changes are accompanied with rapid modulation of gene expression, thus implicating miRNAs. Here we performed deep-sequencing studies to identify shared regeneration miRNAs among zebrafish caudal fins, axolotl forelimbs and *Polypterus* pectoral limbs. Real-time Q-PCR analysis confirmed the evolutionarily conserved miR-21 is one of the most highly upregulated miRNAs in response to injury. *In situ* hybridization studies in zebrafish caudal fins reveal miR-21 expression is localized to the basal-epithelial tissue layer and distal blastemal cells. Experimental depletion of miR-21 levels with antisense oligonucleotides culminated in regenerative outgrowth and patterning defects in all three animal systems. Furthermore, we show in the zebrafish that miR-21 is essential to activate blastema formation and cell proliferation. Using an integrated bioinformatics approach, we identified *bmp3, timp3* and *fgf20a* as miR-21 putative target genes. Collectively, our studies implicate miR-21 as a key component of a miRNA genetic circuit for repair and regeneration of complex appendage tissues.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

Poster presentation and abstract at the 39th Maine Biological and Medical Sciences Symposium:

MicroRNAs Let-7i and miR-101a are regulated during zebrafish caudal fin regeneration

Stephanie Corriveau[1], Devon Cote[2], Bryan Jennings[1], Carly Langley[1], James Lee[3], Robyn Oster[1], Dylan Plissey[1], Klaas Pruiksma[3], Deborah McGann[3], Rachael Hannah[1,4] and Viravuth P. Yin[4]
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Danio rerio (zebrafish) possess an enhanced capacity to regenerate many tissues relative to mammals. Understanding the cellular and genetic influences for appendage regeneration in zebrafish could provide clues for improving mammal regeneration capacity. MicroRNAs (miRNAs) constitute a class of gene expression regulators with important roles during limb morphogenesis and patterning. In this study, we examined the extent that miRNAs let-7i and miR-101a contribute to appendage regeneration. Caudal fins of adult wild-type zebrafish were amputated 50% from the basal body along the proximodistal axis. Noticeable blastemal formation was detected 2 days post-amputation (dpa). At 4 dpa, images of the regenerating caudal fin showed extensive blastemal formation and regeneration. Regenerated tissues were isolated for RNA extraction, cDNA synthesis, and subjected to real-time QPCR studies. MiR-101a exhibited increased expression in regenerating tissues at 2 dpa with a fold change of 16, when compared to uninjured tissues. Conversely, let-7i expression decreased at 2, 4, and 14 dpa (fold change 4.7, 3.2 and 3.1, respectively). These data suggest that miRNAs are rapidly modulated in response to injury, implicating important roles during blastemal formation and tissue outgrowth. In future studies, we will manipulate levels of let-7i and miR-101a and quantify changes in regenerative capacity to better understand miRNA impact on appendage regeneration.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

40th Maine Biological and Medical Sciences Symposium (abstract):

MicroRNA Control of Appendage Regeneration

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The long-range goal of this proposed research is to dissect the molecular regulation of vertebrate limb regeneration, and apply this information toward designing therapies that restore regenerative responses in humans. The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million US citizens. Development of therapies to restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. While this capacity is limited only to the very distal tips of digits in mammals, adult teolost fish and urodele amphibians have championed regeneration of entire appendages, replacing bone, connective tissue, epidermis, nerves, blood vessels and pigment cells. The key feature that underscores appendage regeneration is the formation of the blastema, a highly proliferative progenitor tissue that arises through dedifferentiation of existing cells. Our goal is to identify a core genetic signature that regulates the formation and maintenance of the blastema by comparing high-throughput RNA sequencing (RNA-Seq) datasets from regenerating axolotl forelimbs, bichir pectoral fins and zebrafish caudal fins.

Initiation and progression of appendage regeneration involves modulating multiple genetic programs through regulatory factors. MicroRNAs (miRNAs) are short highly conserved non-coding genes that suppress expression of target genes and thereby control multiple genetic programs. Given the important regulatory roles of miRNAs, and evolutionary conservation, we hypothesize that differentially expressed miRNAs define a conserved genetic regulatory circuit important for appendage regeneration. We found six upregulated and six down-regulated miRNAs common to all three-model systems. The most highly up-regulated miRNA in the three models was miR-21. We are currently analyzing corresponding mRNA-Seq data to find candidate target genes for miR-21 and the other commonly expressed miRNAs. One promising candidate gene is the matrix metalloproteinase inhibitor, *reck*, that is commonly down-regulated in axolotl and bichir and a known miR-21 target gene in human glioblastoma cell lines.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

5th Biennial National IDeA Symposium of Biomedical Research Excellence (abstract):

MicroRNA Control of Appendage Regeneration

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Background and Objective: Our long-range goal is to dissect the molecular regulation of vertebrate limb regeneration, and apply this information toward designing therapies that restore regenerative responses in humans. Development of therapies to restore regenerative capacity first requires an understanding of gene regulatory networks controlling this biology. While this capacity is limited only to distal tips of digits in mammals, adult teleost fish and urodele amphibians have championed regeneration of entire appendages. The key feature of appendage regeneration is the formation of the blastema, a highly proliferative progenitor tissue that arises through dedifferentiation of existing cells. Our goal is to identify a core genetic signature that regulates the formation and maintenance of the blastema by comparing gene expression profiles in regenerating axolotl forelimbs, bichir pectoral fins and zebrafish caudal fins. Given the important regulatory roles of microRNAs and evolutionary conservation, we hypothesize that microRNAs define a conserved genetic regulatory circuit important for appendage regeneration.

Methods: Illumina RNA sequencing of small RNAs and mRNAs, qPCR validation and anti-miR knockdown.

Results: We found six up-regulated and six down-regulated microRNAs common to all three model systems. MiR-21 was consistently up-regulated and anti-miR-21 knockdown inhibited regeneration in each model.

Discussion and Conclusions: MicroRNAs, such as miR-21, are required for appendage regeneration and appear to constitute a conserved regulatory circuit for regeneration.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

11th International Conference on Zebrafish Development and Genetics (abstract):

Regulation of Zebrafish Fin Regeneration by miR-21

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Appendage regeneration is defined by the transformation of quiescent, differentiated tissues into highly proliferative and regenerative blastemal cells. These dramatic cellular changes are accompanied with rapid modulation of gene expression, thus implicating miRNAs. Here we performed deep-sequencing studies to identify shared regeneration miRNAs among zebrafish caudal fins. Real-time qPCR analysis confirmed miR-21 is one of the most highly upregulated miRNAs in response to injury. Subsequent qPCR analysis shows this upregulation in all five zebrafish fin types. *In situ* hybridization studies in zebrafish caudal fins reveal miR-21 expression is localized to the basal-epithelial tissue layer and distal blastemal cells. Experimental depletion of miR-21 levels with antisense oligonucleotides culminated in regenerative outgrowth and patterning defects in all fin types. Furthermore, we show in the zebrafish that miR-21 is essential to activate blastema formation and cell proliferation and depletion of this microRNA effects multiple signaling pathways. Using an integrated bioinformatics approach, we have identified *fgf20A, bmp3, and timp3* as miR-21 putative target genes. Collectively, our studies implicate miR-21 as a key component of a miRNA genetic circuit for repair and regeneration of complex appendage tissues.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

11th International Conference on Zebrafish Development and Genetics (abstract):

Genetic determinants of positional memory during appendage regeneration

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In the United States alone, almost two million people live with limb loss, primarily the result of dysvascular pathology or trauma associated amputations. Amputees suffer from significantly lowered health status, as well as increased rates of mortality; therefore, it is critical to elucidate mechanisms that promote successful limb regeneration. While humans possess limited regenerative capacity, the zebrafish displays a remarkable ability to completely regenerate damaged or lost appendages; however the mechanisms governing this process are not completely understood. During appendage regeneration, an information gradient along appendage axes is believed to regulate positional memory – the meticulous and appropriate replacement of complex tissues. Because both microRNAs (miRNAs) and retinoic acid (RA) are recognized to regulate appendage regeneration, we examined the gradient expression of miRNAs and components of the RA signaling pathway in tissues isolated from three planes along the proximal-distal (P/D) axis in the zebrafish caudal fin. Initial microarray analysis identified miRNAs with significant changes in expression during regeneration. Among these, miR-21 revealed the most dramatic upregulation. qPCR studies demonstrated that expression of miR-21 increases in a gradient along the P/D axis in both uninjured and regenerate fins. In contrast, two components of the RA signaling pathway, RALDH-2 and RXR-αa, are downregulated along the same gradient. The inverse correlation between expression of miR-21 and RA pathway members suggests that miR-21 may play a role in modulating RA signaling. This hypothesis is supported by previous experiments, which demonstrated that the RA co-receptor RXR-Δ is a direct target of miR-21, and that zebrafish caudal fins treated with anti-miR-21 are characterized by proximalization of structures during regeneration, reflecting patterns observed with RA treatment. Our results suggest that miR-21 regulates positional

memory through targeting key components of the RA signaling pathway. This study presents important preliminary data contributing to our understanding of successful limb regeneration.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

Manuscript in submission:

A Conserved Gene Regulatory Circuit During Limb/Appendage Regeneration in Three Vertebrates

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Although regeneration of limb and appendage tissues has been studied in lower vertebrates since the 1600s, we know very little about the genetic circuits that regulate this process. The defining feature of limb/appendage regeneration is blastema formation. This mass of dedifferentiated, proliferative mesenchyme emerges after limb or fin amputation and serves as progenitor tissue for lost structures. Here we used RNA-sequencing technology to profile noncoding microRNAs and mRNAs during blastema formation in three different adult systems; the zebrafish caudal fins, axolotl forelimbs and Polypterus pectoral fins. We identified five up-regulated and five down-regulated common miRNAs, of which, miR-21 was most highly up-regulated. We show and validate expression changes of candidate miR-21 target genes, including the known tumor suppressor, *pdcd4*. In addition, we performed *de novo* genome assembly for Polypterus and axolotl and identified upregulation of known blastemal associated markers. These studies reveal shared regeneration gene regulatory networks among three animal systems separated by millions of years in evolution.

Manuscript in preparation:

The Evolutionarily Conserved microRNA-21 Controls Appendage and Limb Regeneration

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Although regenerative capacity is evident throughout the animal kingdom, it is not equally distributed throughout evolution. For instance, complex appendage and limb regeneration is muted in mammals but enhanced in amphibians and zebrafish. How and why certain organisms are better equipped to replace missing or damaged tissues has perplexed biologists. The distinguishing feature of animals endowed with the capacity for limb/appendage regeneration is the formation of a dedifferentiated, highly proliferative tissue termed, blastema. Here we employ next-generation sequencing to identify and functionally define miRNAs important for injury-induced blastema formation in three different animal systems: zebrafish, axolotl and Polypterus. We found that miR-21 is the most highly upregulated miRNA in injured zebrafish and Polypterus fins and axolotl forelimbs. Notably, genetic depletion of miR-21 function culminated in regeneration defects, including inhibition of blastema formation and patterning defects of the regenerated tissue. We demonstrate that the extent of miR-21 mediated control of Fibroblast growth factor (fgf20a) activity determines whether the regenerative genetic pathway is activated or inhibited. This work reveals that the miR-21/fgf20a circuit constitutes a key component of the regenerative circuit that was conserved throughout evolution.

Patent Application filed:

Zasloff MA, Strange K and **Yin VP.** Stimulation and Enhancement of Regeneration of Tissues. US PCT/US13/77118. 2013 December 20.

CONCLUSIONS:

In order to develop potential therapies to restore and/or augment human limb regeneration, we must first understand the molecular regulation of appendage regeneration in vertebrates that have retained enhanced regenerative capacity during evolution. Two critical limitations have impeded progress in this area: 1) lack of diverse experimental animals has precluded the powerful comparative approaches that have vertically advanced other fields of regenerative medicine such as stem cell biology; 2) unbiased functional genomic approaches have not been fully exploited. The experimental design we advance in this proposal integrates two innovative approaches to address these issues, and distinguishes this work as a unique and complementary extension of current projects in TATRC's portfolio. We have and will continue to use a novel comparative approach employing phylogenetically diverse experimental organisms, and adopt unbiased systems-level approaches to identify and dissect the gene networks initiating and maintaining regenerative responses in vertebrate limbs.

The scientific knowledge revealed during this funding period highlights the importance of miRNAs as conserved, global regulators of regenerative capacity. In particular, our functional analysis of miR-21 indicate this is a critical regulator of blastema formation across animal systems that have diverged from a common ancestor ~400 Million years ago in evolution. Future comparative studies to understand the detailed cellular and molecular mechanism of action for miR-21 will be important to identify additional therapeutic targets to augment regenerative capacity in humans.

REFERENCES: N/A

APPENDICES: N/A

SUPPORTING DATA: N/A